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THE VELIGER

NOTES, INFORMATION & NEWS

A Useful Marker for the Study of Neural Development in Cephalopods

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In cephalopods, there is no suitable marker that visualizes three-dimensional neural patterns in the preserved embryos. Development of the nervous system has been studied using conventional histological techniques (Meister, 1972; Marquis, 1989). Silver impregnation and methylene blue staining have been used in anatomical studies of the nervous system in cephalopods (Stephens, 1971) but the former are applicable only to late embryonic stages (Martin, 1977) and the latter, only to living neurons. Cobalt backfilling (Budelmann & Young, 1987) and DiI injection (Robertson et al., 1993) only label local neuronal patterns. We tried whole mount immunostaining of cephalopod embryos and hatchlings with commercially obtainable monoclonal antibodies and found acetylated α-tubulin a suitable immunohistochemical marker to visualize the overall pattern of developing neurons.

We used four sepiids, *Idiosepius paradoxus* Ortmann, 1881; *Euprymna morsei* (Verrill, 1881); *Sepia lycidas* Gray, 1849; *Sepiella japonica* Sasaki, 1929; two teuthoids, *Loliolus japonica,* Hoyle, 1885; *Todarodes pacificus* Steenstrup, 1880; and an octopod, *Octopus ocellatus* Gray, 1849 (Octopoda). Dechorionated embryos at various stages of neurogenesis and hatchlings were fixed in 4% paraformaldehyde (PFA) dissolved in phosphate buffered saline (pH 7.6) (PBS) for 12–24 hr at 4°C. Samples were washed with PBS, dehydrated in a methanol series, and stored in 80% methanol at −20°C. Some samples were also fixed in Bouin's solution/seawater, dehydrated with an ethanol series, and stored in 70% ethanol at room temperature. The stored samples to be immunostained were placed as a whole, or after dissection into a few pieces, in ice-cold 50% dimethyl sulfoxide (DMSO)/methanol with 10% hydrogen peroxide for 5 min. They were incubated for 30 min at 4°C in the DMSO solution with 1% Triton X-100, washed with Tris-buffered saline (TST; 20 mM Tris-HCl, pH 8.0, 150mM NaCl, 0.1% Triton X-100) containing 5% DMSO, and blocked with 5% non-fat dry milk (TSTM) overnight at 4°C. The specimens were incubated with anti-acetylated α-tubulin antibody (Sigma) diluted 1:750–1000 in TSTM for 2–4 d at 4°C (Gianni & Fuller, 1985). After being washed with TSTM, some samples were incubated in pre-diluted goat anti-mouse antibody conjugated to peroxidase (Envision+, DAKO) for 12–24 hr at room temperature, washed with TSTM, immersed in ice-cold 3,3′-diaminobenzidine (DAB) (1 mg/ml TST) for 1 hr, and reacted by adding hydrogen peroxide (0.01%) for 5–20 min in the dark. The other samples were stained with ABC high-HRP immunostaining kit (TOYOBO) according to the standard protocol.

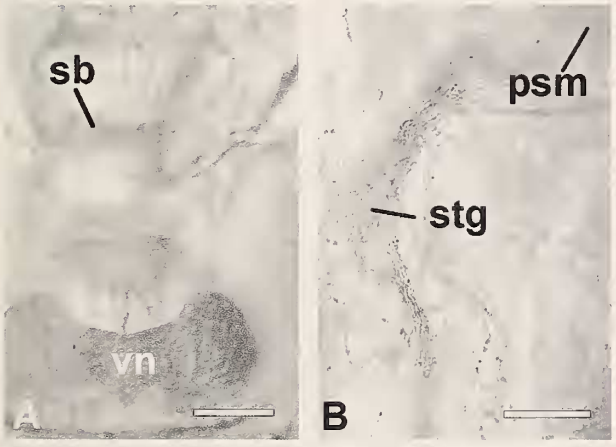

Figure 1. Neuropiles in whole-mount of specimens immunostained with acetylated α-tubulin antibody. A. The vertical lobe (vn) and the superior buccal lobe (sb) in a late embryo of *Sepia lycidas*. The cranial cartilage is removed. Scale bar = 300 μm. B. The stellate ganglion (stg) and the posterior subesophageal mass (pms) in an *Idiosepius paradoxus* hatchling. Scale bar = 30 μm.

Anti-acetylated α-tubulin clearly stained peripheral nerve fibers as well as neuropiles in the brain (Figure 1A). It also stained the epidermal cilia, lateral lines, ectodermal photosensitive vesicles, Kölliker's canals of the statocysts, and olfactory organs. The antibody recognized neurons even in Bouin-fixed specimens that had been stored for 3 yr in ethanol, though not always consistently. As for the secondary antibodies, Envision+ (DAKO) was slightly more effective than ABC high HRP (TOYOBO) kit. The intensity and the extent of visualization depended on the limit of penetration of the antibodies. In small specimens, such as the embryos and hatchlings of *I. paradoxus* and the embryos of *E. morsei,* all neuronal elements, i.e., peripheral nerves and neuropiles in the brain, were observable in the samples mounted as a whole (Figure 1B). In larger specimens, such as *O. ocellatus, S. lycidas, S. japonica,* and *L. japonica,* dissection was necessary before immunostaining permitted visualization of the neuropiles in the deep portion of the brain and all the peripheral nerve fibers in the body. We tested two other monoclonal antibodies, anti-neurofilament 200 (Sigma)

and anti-HRP (Sigma) (Jan & Jan 1982), but they did not recognize any neuronal elements.

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Crepidula dilatata Lamarck, 1822, Truly Living in the Southwestern Atlantic

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Crepidula fecunda Gallardo, 1979, was described from Bahía Chinquihue (41°31′S–73°03′W) in the Chilean Pacific. The distribution ranges from the type locality, 41°S, to 45°S off the Chilean coast. According to Gallardo (1979), *C. dilatata* Lamarck, 1822, can only be differentiated by its direct development and the presence of embryos consuming nurse eggs. Adult morphological features are identical. Therefore, earlier records referring to the presence of *C. dilatata* in the Atlantic coast of South America (Parodiz, 1939) need validation. The type locality of *C. dilatata* remains unknown. Mermod (1950) mentioned in a commented list of the types from Lamarck's collection, the Western coast of South America as a probable type locality. Gallardo (1979) recorded *C. dilatata* Lamarck, from 21°11′S to 43°47′S. He also stated (in Spanish in the original): "It is probable that future studies including developmental stages, will expand this distribution particularly towards the Argentine Atlantic coast."

This note confirms the presence of *C. dilatata* (Figures 1–9) in Argentine waters and restricts *C. fecunda* to Chile.

Egg capsules and adult males and females were collected from Bahía Ensenada, Ushuaia (~55°S) by SCUBA diving in 3–4 m depth, attached to the root of the common kelp *Macrocystis pyrifera* (Linnaeus); Punta Peñas, Puerto San Julián (49°15′S–67°39′W) in 2 m depth; and Punta Dos Hermanas, Puerto Deseado (47°10′S; 2–3 m depth) in Santa Cruz province; and several localities around Golfo Nuevo (~42°30′S) in Chubut province (subtidal). All collections were made during February 2000.

We studied more than 100 brooding females (voucher material was deposited in Museo Argentino de Ciencias Naturales, number MACN 33901). Most females were brooding egg masses at advanced stages of embryonic development, containing embryos and uncleaved nurse eggs or crawling juveniles. This fact confirms the presence of *C. dilatata* in the southern Atlantic, and as far as we observed, restricts *C. fecunda* to the Pacific.

The observed material was completely homogeneous, with only one developmental mode characterized by the presence of nurse eggs. Each egg capsule (n = 150) contained 203–375 eggs (mean = 303, SD = 54) with only two to 12 developing embryos, representing as an average 2.4% of the initial egg number. The average uncleaved egg diameter was 214 μm (SD = 13 μm, n = 72). The egg diameter distribution adjusted to a normal distribution with a single mode was 212 μm. Hatching occurred at a crawling juvenile stage. The egg capsule size averaged 3873 μm in length and 3954 μm in width (SD = 648 and 527 μm, respectively). Brooding females measured 11–32 mm (mean = 22 mm) in shell length, but in this protandric species the loss of penis can be already observed at 11 mm of shell length. Males (with a penis) measured 7–19 mm of shell length.

The Argentine material agrees with Gallardo's (1976,